INTERNATIONAL SEARCH REPORT

941/163/998

International Application No
PCT/CA 99/00813

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 A61K39/104

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 97 01647 A (TRUSTEES OF HEALTH & HOSPITALS) 16 January 1997 (1997-01-16) cited in the application the whole document ---	1-5,8, 11-16
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Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2000	17/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Knehr, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9701647 A	16-01-1997	AU 6404196 A	30-01-1997
WO 9820157 A	14-05-1998	US 5994066 A	30-11-1999
		AU 4859897 A	29-05-1998
		EP 0943009 A	22-09-1999
		NO 991976 A	02-07-1999

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INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

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Smart & Biggar
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650 W. Georgia St., Suite 2200
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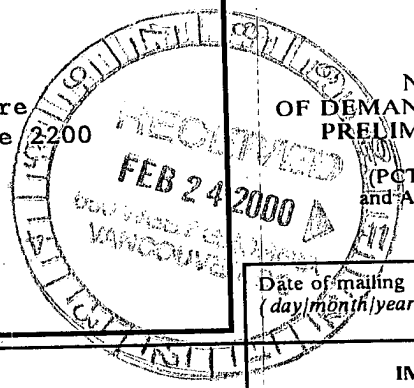

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*	18.02.00
Applicant's or agent's file reference: 80472-5	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA 99/00813	03/09/1999	03/09/1998

Applicant: THE UNIVERSITY OF BRITISH COLUMBIA et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

07/02/2000

2. This date of receipt is:

- [X] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

[ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

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European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	WAHRHEIT D Tel. (+49-89) 2399-2651

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NOTIFICATION OF ELECTION

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Date of mailing: 16 March 2000 (16.03.00)	
International application No.: PCT/CA99/00813	Applicant's or agent's file reference: 80472-5
International filing date: 03 September 1999 (03.09.99)	Priority date: 03 September 1998 (03.09.98)
Applicant: MAHENTHIRALINGAM, Eshwar	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

07 February 2000 (07.02.00)

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The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

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(30) Priority Data:

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60/099,116	3 September 1998 (03.09.98)	US

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; IRC Room 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3 (CA).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: MAHENTHIRALINGAM, Eshwar [CA/GB]; Cardiff School of Biosciences, Cardiff University, P.O. Box 915, Cardiff CF1 3TL (GB).

(74) Agents: ROBINSON, J., Christopher et al.; Smart & Biggar, 2200–650 West Georgia Street, Box 11560, Vancouver, British Columbia V6B 4N8 (CA).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR THE IDENTIFICATION AND SPECIATION OF BACTERIA OF THE *BURKHOLDERIA CEPACIA* COMPLEX

(57) Abstract

Identification and speciation of bacteria of the *Burkholderia cepacia* complex in a sample can be accomplished by: a) obtaining nucleotide sequence information for the *recA* gene in bacteria of the *Burkholderia cepacia* complex found in the sample; and b) comparing the nucleotide sequence information obtained for the *recA* gene in bacteria of the *Burkholderia cepacia* complex found in the sample with a standard library of nucleotide sequence information comprising standard nucleotide sequence information for at least three species of bacteria of the *Burkholderia cepacia* complex. Preferably, the nucleotide sequence information is obtained by evaluation of restriction fragment length polymorphism (RFLP). Other techniques for obtaining sequence information can also be used, including base–by–base determination of the sequence of the region of interest, sequence–specific oligonucleotide hybridization probes, and ligation techniques. Universal primers which can be used for amplification of all known members of the *Burkholderia cepacia* complex, and genomovar–specific primers which can be used for selective amplification of the *recA* gene from bacteria of one genomovar provide alternative analytical modalities. Speciation of bacteria of the *Burkholderia cepacia* complex can be used as a basis for administration of a vaccine specific to the flagellin of the bacteria, since it is shown that this flagellin is conserved across members of genomovar III, subgroup RG–B.

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CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

5' - TCG AGA CGC ACC GAC GAG - 3' SEQ ID No. 33

PCR product expected from *B. cepacia* strains of genomovar III = 378 bp.

Additional sequencing of the complete *recA* gene from *B. cepacia* complex strains M36, M54, Ral-3 and *B. pyrrocinia* LMG 14191^T or partial sequence analysis of PCR amplicons derived from strains ATCC 29464, ATCC 53617, ATCC 39277, ATCC 49709 and ATCC 53266 was performed. The phylogenies determined using partial sequencing of this type were identical to those determined using the full sequences (Fig. 2), however, two additional clusters, denominated as RG-C and RG-D were identified. Cluster RG-C was a novel group consisting of biocontrol strains Ral-3, ATCC 53266 and M54. Comparative alignment of the *recA* sequence from Ral-3 and M54 with all other complete *B. cepacia* sequences enabled the design of RG-C specific primers with the following sequences:

Forward Primer:

GTCGGGTAAAACCACGTG SEQ ID No. 39

Reverse Primer:

TCCGCAGCCGCACCTTCA SEQ ID No. 40

B. cepacia biocontrol strains BC-B, BC-F and AMMD all tested positive with this RG-C primer set. Thus, these primers can be used in analytical schemes for the presence of such primers, and also could be used for screening isolates for biocontrol properties.

A second novel *recA* group, RG-D, was identified which includes *B. pyrrocinia* LMG 14191^T and ATCC 32977, a strain of *B. cepacia* which produces the antibiotic xylocladin. This group is also shown in Fig. 2.

In addition to a method for identification and specification of bacteria of the *B. cepacia* complex, the invention also provides reagents and kits suitable for carrying out this method. The reagents are generally polynucleotide primers or probes which bind to the *recA* gene of one or more strains of bacteria of the *B. cepacia* complex. One subset of the reagents of the invention are non-specific primers, such as used in Example 4 below, which are complementary to conserved regions found identically in strains of bacteria of the *B. cepacia* complex for which the sequences are given. A second subset of reagents in

accordance with the invention are primers/probes which can be used to selectively amplify and/or detect one genomovar of bacteria of the *B. cepacia* complex. The reagents of the invention may have a detectable or capturable label, for example a radioactive or fluorescent label or biotin, incorporated therein to facilitate evaluation of nucleotide sequence information.

Either of these types of primers/probes may be packaged in a kit with suitable reagents. These reagents may include discriminatory restriction enzymes, which are capable of producing distinctive fragment patterns to permit speciation of a bacteria-containing sample, or reagents suitable for PCR, nucleic acid sequencing and the like.

Once the species of a sample bacterium of the *B. cepacia* complex is determined using the method of the invention, it may be desirable (particularly where the bacteria is a member of an epidemic strain) to be able to provide a therapeutic agent which is effective in treating or preventing infection. Thus, the present invention further provides a vaccine composition based upon the antigenic properties of the flagellin of epidemic strains of *B. cepacia* complex for use in treating infections caused by certain species of the *B. cepacia* complex.

The use of flagellins as an antigen for vaccine purposes has been proposed in a variety of instances because of their location on the outside of bacterial cells. In the case of *B. cepacia* complex, however, Hales et al., *J. Bacteriol.* 180: 1110-1118 (1998), have reported that the flagellin gene (fliC) is "highly variable" and suggest its utilization as a biomarker for epidemiological and phylogenetic studies of *Burkholderia cepacia*. Such variability is inconsistent with the normal requirements that a vaccine antigen be highly conserved, such that its will be generally effective against variants of the target species. Thus, it was quite surprising to find that the subset of *B. cepacia* complex which is most transmissible have highly conserved flagellin genes which is suitable for use as a vaccine.

A total of 30 strains of bacteria of the *B. cepacia* complex were classified using the speciation method of the invention into groups based on the sequence of the *recA* gene, and were in addition characterized with respect to the BCESM and *cbl*A markers for highly transmissible strains of *B. cepacia*. As reflected in Table 1, a substantial portion of the genomovar III strains which were positive for one or both of these markers produced a

single RFLP pattern (Fig. 3, pattern G) after treatment with the restriction endonuclease *HaeIII*.

Exemplary sequences and a consensus sequence for the *B. cepacia* flagellin gene, which encodes the major subunit protein of the bacterial flagellum of *B. cepacia*, have been described in the literature by Hales et al. (supra). Using the same primers described by Hales, it has been determined that the flagellin genes of *B. cepacia* strains of *recA* type III-G (genomovar III, with *recA* RFLP pattern G) are highly conserved and do not vary considerably in DNA sequence. This indicates that the protein is also highly conserved in its structure and sequence, and thus is suitable for use as an antigen for development of vaccines against the most problematic strains in patients with cystic fibrosis (CF).

In contrast, the flagellin gene of *B. multivorans* strains of recA type F (genomovar II), which appear less problematic in patients with CF and do not generally spread among patients, have flagellin genes which are highly variable in sequence. These data suggest that with *B. multivorans* strains, a vaccine based on the flagellum may not protect against infection with all strains types as has been the case with the bacterium *Pseudomonas aeruginosa* in CF. Thus, the observation, that the flagellin gene is actually highly conserved in the most devastating epidemic *B. cepacia* strains infecting patients with CF is apparently unique to this subset of the species of the *B. cepacia* complex.

The observation that the flagellin gene is conserved among *B. cepacia* strains which are epidemic amongst patients with CF permits the development of a vaccine based on the encoded protein antigen. The vaccine can be prepared in a variety of ways. First, protein can be purified from bacterial strains representative of this group to obtain a purified antigen. Methods for purification of flagellin from bacteria are known in the art, and can be applied to recovery of purified flagellin from epidemic strains of *B. cepacia.* Purified antigen is then used as a vaccine, with or without an adjuvant. Vaccines of this type are generally administered by subcutaneous or intramuscular injection, although other routes of administration may also be suitable. Therapeutically effective levels and frequency of vaccine administration are determined by routine monitoring of antibody titers.

In addition to the use of purified flagellin isolated directly from bacteria, it will be appreciated that the same protein, or an immunologically effective portion thereof may

epidemic amongst patients with CF (Mahenthiralingam et al., *J. Clin. Microbiol.* 34: 2914-2920 (1996) and encode the BCESM (Mahenthiralingam et al., *J. Clin. Microbiol* 35: 808-816 (1996). The separate classification of these three strains based on the recA suggest that they may constitute a new species/genomovar group within the *B. cepacia* complex.

EXAMPLE 4

To obtain nucleotide sequence information about the *recA* genes of additional strains of bacteria of the *B. cepacia* complex (Table 1), samples of each strain were amplified using the following primers:

Forward Primer (BCR1)

TGACCGCCGAGAAGAGCAA SEQ ID No. 3

Reverse Primer (BCR 2)

CTCTTCTTCGTCCATCGCCTC SEQ ID No. 4

using a standard polymerase chain reaction mixture of 25 microlitres in volume (described in Mahenthiralingam et al., *J. Clin. Microbiol.* 35: 808-816 (1996)) containing 1.5 mM $MgCl_2$ and 10-20 ng of *B. cepacia* DNA. Amplification was performed as follows: 30 cycles of 1 min. at 94°C, 1 min. at 56°C , and 2 min. at 72°C, follow by a final 6 min. cycle at 72°C. This resulted in the amplification of a 1 kb DNA band corresponding to the *recA* gene of the *B. cepacia* strain being tested.

Several restriction enzyme were screened for their ability to reveal DNA sequence variation in this amplified gene which would be suitable for speciation of *B. cepacia*. The enzymes *Hae* III and *Alu* I were found to be suitably discriminatory. The restriction fragments produced by the enzyme *Hae* III were separated by agarose gel-electrophoresis, and the detected restriction fragment length polymorphisms (RFLPs) demonstrated that genomovar specific RFLPs could be generated using this approach. Representative patterns are shown in Fig. 3. (Bv = *B. vietnamiensis*, or genomovar V; Gv I = genomovar I; Bm = *B. multivorans* or genomovar II; Gv III = genomovar III and Gv IV = genomovar IV). This same approach has been applied to a panel of strains which are representative of all five genomovars of *B. cepacia* and been found to be able to distinguish strains of each genomovar (Table 1). This technique has also been applied to additional strains, and been

CTCTTCTTCGTCCATCGCCTC. SEQ ID No. 4

7. The method of claim 5, wherein the PCR amplification is carried out using the following primers:

Forward Primer

TGCGGATGGGCGACGGCG SEQ ID No. 20

Reverse Primer

CAGTTCTGTCGCTTGATCG. SEQ ID No. 21

8. A composition comprising a pair of polynucleotide primers effective to amplify the *rec*A gene of bacteria of the *Burkholderia cepacia* complex, wherein the primers are effective to amplify at least a diagnostic portion of each of the genes given by SEQ ID Nos. 1, 2 and 5-19.

9. The composition of claim 8, wherein the polynucleotide primers have the sequences:

Forward Primer

TGACCGCCGAGAAGAGCAA SEQ ID No. 3

Reverse Primer

CTCTTCTTCGTCCATCGCCTC. SEQ ID No. 4

10. The composition of claim 8, wherein the polynucleotide primers have the sequence:

Forward Primer

TGCGGATGGGCGACGGCG SEQ ID No. 20

Reverse Primer

CAGTTCTGTCGCTTGATCG. SEQ ID No. 21

11. A kit for speciation of bacteria of the *Burkholderia cepacia* complex, comprising, in packaged combination, a pair of polynucleotide primers in accordance with any of claims 8 - 10, and a discriminatory restriction endonuclease.

12. The kit of claim 11, wherein the restriction endonuclease is *HaeIII* or *AluI*.

13. A composition comprising a genomovar-specific primer pair effective under stringent PCR conditions to produce amplification products by amplification of at least a portion of the *recA* gene of bacteria belonging to one genomovar of the *B. cepacia* complex, but not to produce amplification products from bacteria belonging to other genomovars.

14. The composition according to claim 13, wherein the genomovar-specific primer pairs are selected from among the following primer pairs given by Seq ID Nos. : 22 and 23, 24 and 25, 26 and 27, 28 and 29, 30 and 31, or 32 and 33.

15. A kit for speciation of bacteria of the *Burkholderia cepacia* complex, comprising, in packaged combination, a pair of genomovar-specific polynucleotide primers in accordance with claims 13 or 14 and a discriminatory restriction endonuclease.

16. The kit of claim 15, wherein the restriction endonuclease is *HaeIII* or *AluI*.

17. A vaccine composition for treatment or prevention of infection with bacteria of the *Burkholderia cepacia* complex, wherein the bacteria is a member of genomovar III and has a nucleotide sequence for the recA gene which produces a G-type RFLP pattern when analyzed with the restriction enzyme *HaeIII*, and wherein the vaccine composition comprises flagellin or a flagellin-derived antigen or a polynucleotide encoding flagellin or a flagellin-derived antigen.

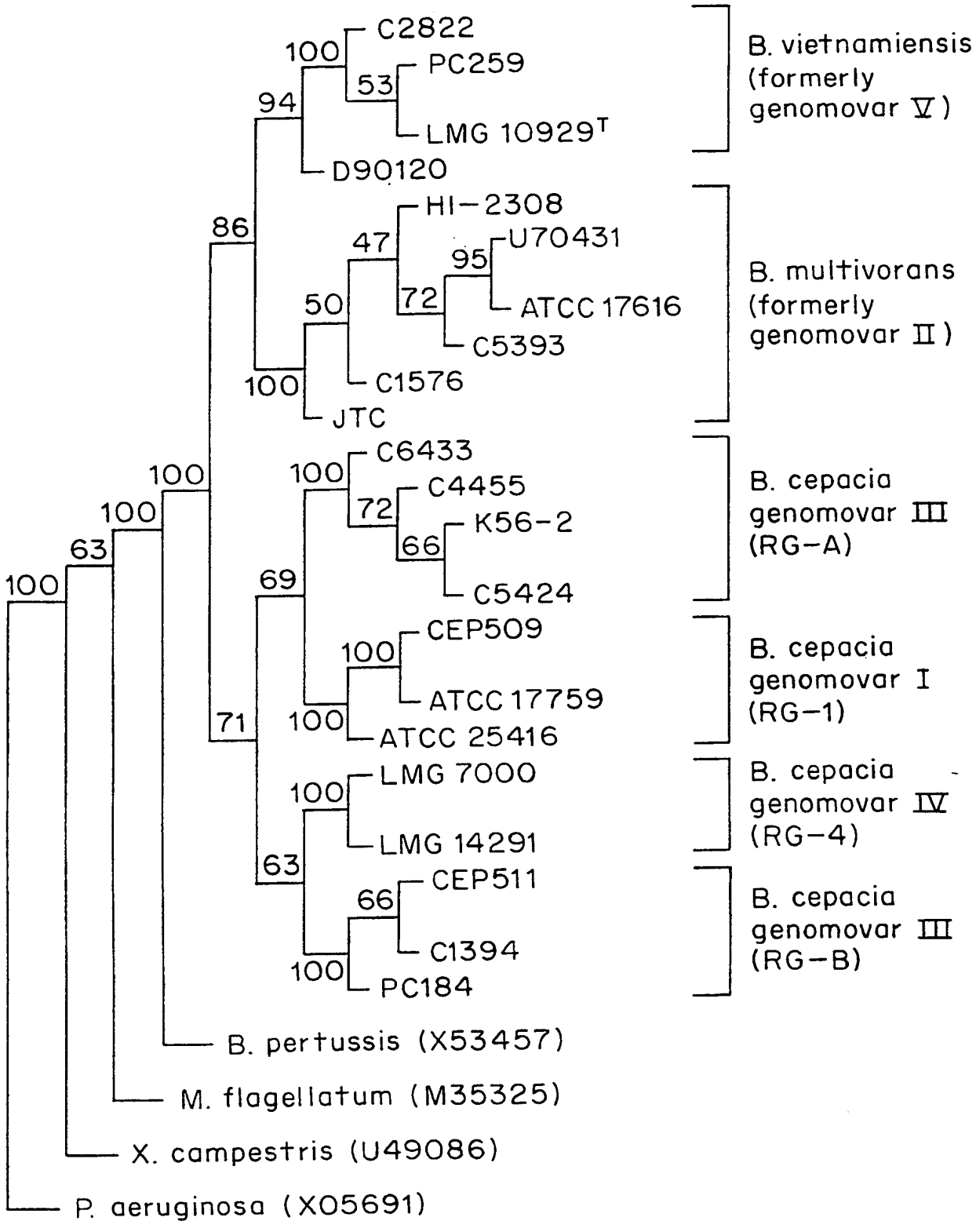
C2822
PC259
LMG 10929T
D90120
HI-2308
U70431
ATCC 17616
C5393
C1576
JTC
C6433
C4455
K56-2
C5424
CEP509
ATCC 17759
ATCC 25416
LMG 7000
LMG 14291
CEP511
C1394
PC184
B. pertussis (X53457)
M. flagellatum (M35325)
X. campestris (U49086)
P. aeruginosa (X05691)
B. vietnamiensis (formerly genomovar V)
B. multivorans (formerly genomovar II)
B. cepacia genomovar III (RG-A)
B. cepacia genomovar I (RG-1)
B. cepacia genomovar IV (RG-4)
B. cepacia genomovar III (RG-B)

FIG. 1